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13. ABSTRACT ***(Maximum 200 Words)***

This study set out to evaluate the biological consequences of a shift in the ratio of the serine protease matriptase relative to its cognate inhibitor HAI-1. The goal of this work is to generate data that we believe will assist in evaluating whether the matriptase HAI-1 system plays a role in breast cancer invasion and metastasis. In order to alter protease / inhibitor ratios we proposed to use a tetracycline regulable expression system to either increase matriptase levels by overexpression or reduce HAI-1 levels by expression of a ribozyme targeted to the HAI-1 mRNA. We have shown that at least in MCF-7 cells, increased expression of Matriptase does not have any impact on any of the properties of the cells that we have examined. This leads us to conclude that these cells either make enough Matriptase already, and additional enzyme is irrelevant, that sufficient HAI-1 is made by the cells to inhibit the activity of the extra Matriptase, or that Matriptase is irrelevant to these processes in these cells. Ongoing studies should allow us to distinguish between these possibilities.

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Table of Contents

Introduction:

Matriptase is a serine protease that is made by epithelial cells in many tissues including the mammary gland. In tissue culture systems and normal tissue, it is always co-expressed with its cognate inhibitor KSIP-1, which we now refer to as HAI-1 (Hepatocyte Activator Inhibitor-1). In breast tumors, it appears that this association between the two proteins is somewhat loser and so this project set out to characterize the biological implications of relative over expression of matriptase versus HAI-1. The goal of the research is to provide data that will assist in the evaluation of HAI-1 and matriptase determinations as prognostic markers and matriptase as a target for therapy.

Body:

Progress on Task 1.

In the previous annual report we described our work characterizing the structure of the Matriptase gene, cloning the extreme 5' end of the gene and using the resultant truly full length clone to generate a tetracycline inducible Matriptase expression vector. This vector was then used to generate a series of MCF-7 clones in which the overexpression of Matriptase could be induced by treatment with doxycycline a tetracycline analogue. We described the initial characterization of these clones and that we had experienced some difficulty getting the cells to grow in nude mice.

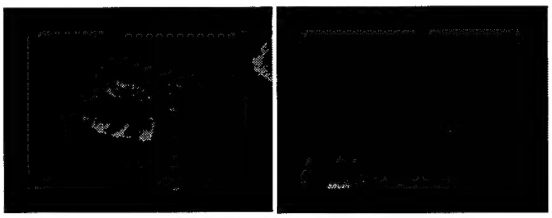
In the last year we have continued to characterize these clones and have conducted a series of additional animal experiments to evaluate the tumorigenicity of the lines.

Cell morphology and structure:

Since the morphology and structure of cells is believed to play a role in the invasive and metastatic behavior, we set out to determine if overexpression of Matriptase had any effect on this aspect of the cells.

Cells were examined for morphological changes after Matriptase induction. No obvious changes in cellular shape were observed at the light microscopy level in induced cells and immunofluorescence microscopy also suggested that Matriptase did not change actin cytoskeletal architecture

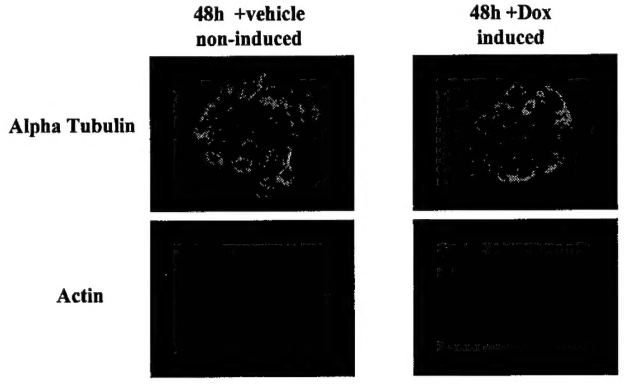
Over-expression of Matriptase does not alter the actin cytoskeleton



M69 (active Matriptase) **actin**

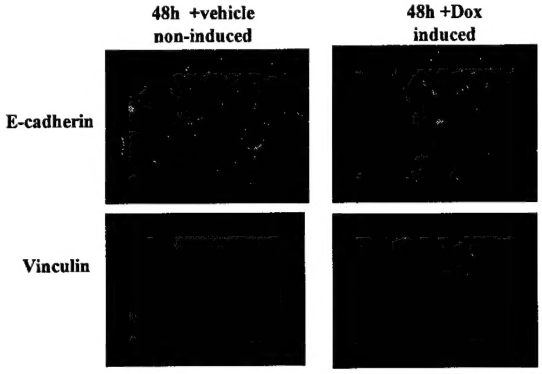
Similarly no alterations were observed in tubulin distribution,

No effect of Matriptase over-expression on actin or tubulin components of the cytoskeleton



or on e-cadherin or vinculin.

No effect of Matriptase over-expression on E-caherin or vinculin expression and distribution



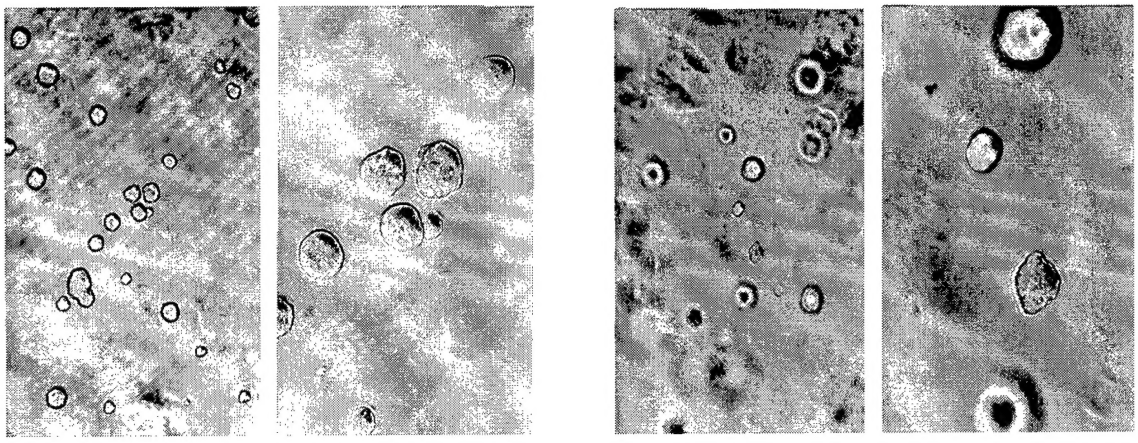
Growth in semi-solid media:

One of the characteristics of highly malignant cells is the ability to grow in semi-solid media – so called anchorage independent growth. There are two basic ways to assess this ability in cells that we have used in the past – growth in matrigel and growth in soft agar. We used both methods to evaluate the anchorage independent growth capacity of the transfectants.

To determine whether Matriptase affected the morphology of cells grown in a three-dimensional matrix, cells were cultured in Matrigel matrix for 5 days, either in the absence or presence of doxycycline to induce the expression of Matriptase. Sub-

confluent cells were removed from tissue culture plastic by trypsin/EDTA treatment and re-suspended in media containing 5% fetal bovine serum to neutralize trypsin activity. After washing once in culture media, 10,000 cells were pelleted by centrifugation at 500g. Media was removed, and cells were resuspended in 0.5 mL of Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA) at 4°C, and plated into a single well of a 24-well plate pre-coated with 0.5mL of solid Matrigel matrix. After solidification of the Matrigel matrix/cell suspension, 1 mL of culture media was added to each well, and cells were incubated under standard culture conditions for 3 days, at which time the medium was changed and the cells were grown for an additional 2 days. To induce the expression of Matriptase in tetracycline-inducible cells, a final concentration of 250ng/mL doxycycline was included in the matrix/cell suspension and culture media. Morphology was assessed by light microscopy, and color images were taken with standard 35mm color slide film.

Media 5 days **250 ng/mL Doxycycline 5 days**

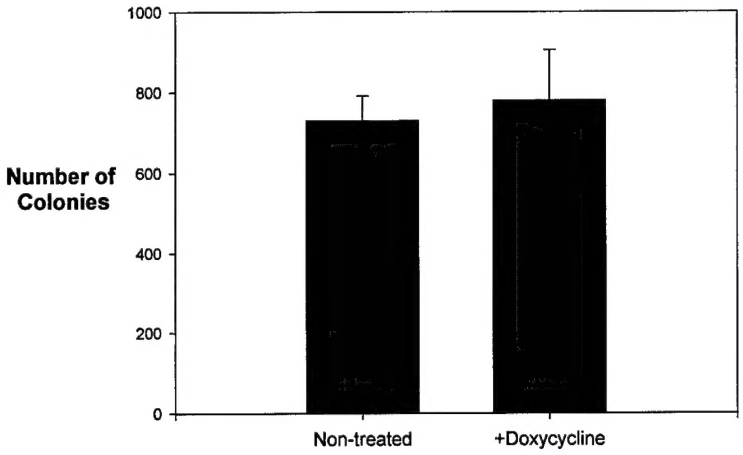


10X **40X** **10X** **40X**

As can be seen, induced and non-induced cells both grew as spherical cell colonies typical of MCF-7 cells (Sommers, et al. 1994). Therefore, Matriptase over-expression did not alter cellular morphology in the context of growth in a solid matrix. This experiment was particularly relevant since Matriptase cleaves substrates that are known to be involved in matrix remodeling and tissue morphogenesis, such as uPA and HGF.

For growth in soft agar, $4X10^3$ cells were plated into each well of a 6-well plate suspended in 0.36% bacto-agar (Becton Dickinson) on a 0.6% agar support, to which doxycycline had been added as appropriate for tet-inducible cells. The cells were grown for 10 days, followed by counting of colonies with an Omnicon 3800 colony counter (Imaging Products International, Inc., Chantilly, VA). All soft agar assays were performed two or three times with similar results. As can be seen from the following

figure, induction of Matriptase expression by treatment with doxycycline had no significant effect on the number of size of the colonies that were formed.



Invasive behavior:

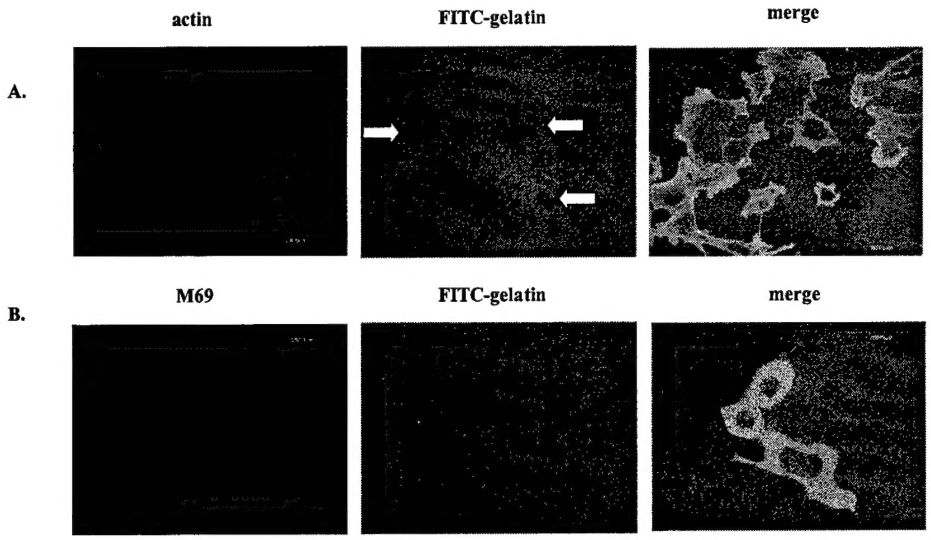
To evaluate the invasive behavior of the cells overexpressing Matriptase we set out to evaluate the clones using several methods. The first we used was the FITC-gelatin degradation assay. In this assay, the ability of cells to digest holes in a thin layer of labeled gelatin is evaluated.

FITC-gelatin degradation assay.

Microscope cover glasses (Fisher Scientific, Pittsburgh, PA) were coated with polylysine by incubation in 5ug/mL polylysine for 20 minutes at room temperature. After washing in PBS, coated coverslips were treated with 0.5% gluturaldehyde for 15 minutes. After further washes in PBS, cover glasses were coated with FITC-conjugated gelatin by incubation in 0.4% FITC-gelatin for 30 minutes. Following coating, cover glasses were treated with 5mg/mL sodium borohydride in PBS for 5 minutes, and rinsed with PBS prior to sterilization in 70% ethanol for 15 minutes. Cover glasses were then rinsed in PBS, incubated for 30 minutes in serum-free IMEM, and cells seeded at approximately 70% cell density. After 24 hours, degradation of the FITC-conjugated gelatin was examined by immunofluorescence microscopy using an Olympus Fluoview confocal microscope attached to an Olympus 1X70 inverted fluorescence scope (Olympus America, Inc., Melville, NY) using the 60X (NA1.4) Olympus objective. Images were captured using the Olympus Fluoview software (Olympus America, Inc.).

In the figure below, MDA-MB-231 cells were used as a positive control, and as can be seen in panel A, areas of reduced green fluorescence are produced under the cells. This is the result of the gelatin being digested by the cells, thereby reducing the signal. The

Matriptase overexpressing cells in panel B do not produce any such areas of clearing suggesting that they are not able to digest the gelatin and reduce the signal. This finding is consistent with the zymography data that was presented in the previous annual report.



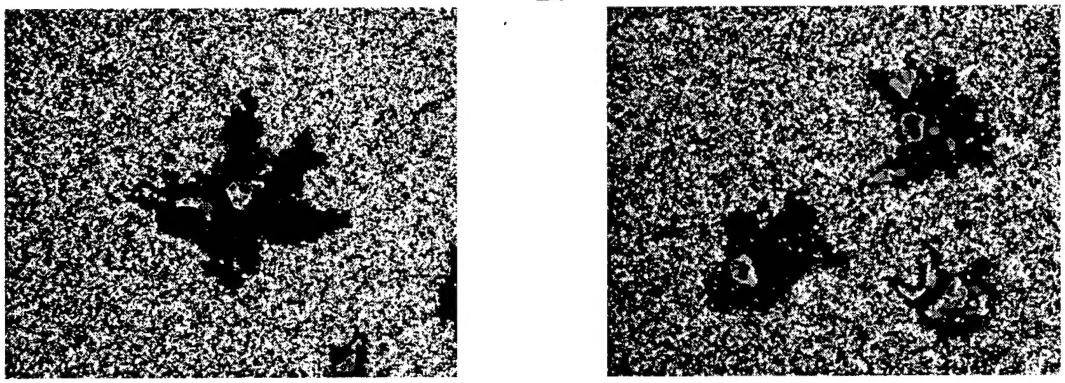
Phagokinetic gold assay:

Next, effects of Matriptase overexpression of the motility of the cells was examined using the phagokinetic gold assay, in which the movement of cells during culture is evaluated by examining the tracks produced on cover slips coated with colloidal gold. Assays were conducted using a modification of the method of Albrecht-Buehler (Albrecht-Buehler, 1977). Briefly, microscope cover glasses (Fisher Scientific, Pittsburgh, PA) were coated with BSA by immersion in a 1% BSA solution, were dipped in 100% ethanol, dried in a hot air stream, and placed in 12-well plates. Colloidal gold was prepared by adding 1.2 mL of 0.1% formaldehyde drop-wise to a boiling gold chloride solution (1.4mM gold chloride and 35mM Na_2CO_3). The hot (80-90°C) gold chloride solution was then placed onto BSA-coated cover glasses and incubated for 20 minutes. After washes in serum-free media, cover glasses were coated with 40ug/mL fibronectin for 1 hour. Cells were trypsinized, counted, and plated at 5,000 cells/well onto the colloidal-gold covered cover glasses. After the allotted time, cells were fixed on cover glasses using 3.7% formaldehyde/0.05% Triton X-100 in PBS for 20 min at room temperature. Cells were then stained and visualized by immunofluorescence for Matriptase, actin, and nuclei (DAPI). Darkfield microscopy was used to visualize tracts made by cells in the colloidal gold. All phagokinetic gold assays were performed at least three times with similar results.

These assays generate data of the kind that is shown below. A field of colloidal gold particles can be seen with areas of clearing that have been produced by the cells migrating around and phagocytosing or moving the particles. The nuclei of the cells

within the area of clearing are visualized by DAPI staining and the area of clearing per cell can be evaluated.

A. **B.**



Panel A shows a typical area of clearing produced by un-induced cells. The cells in panel B were treated with doxycycline and as can be seen, there was no effect on the motility of the cells.

Chemoinvasion assay:

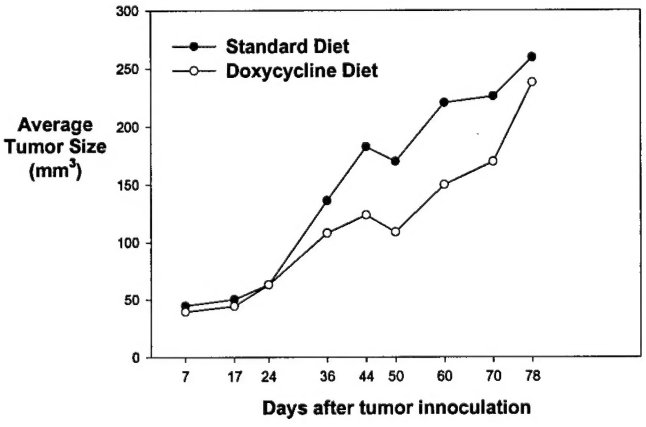
The final assay system we used to evaluate the effects of Matriptase overexpression on the invasive behavior of cells in vitro was the chemoinvasion assay. In this assay, the ability of cells to penetrate an artificial basement membrane is determined. The invasion of cells through a Matrigel-coated polycarbonate membrane with 8um pores was assessed using Biocoat Matrigel invasion chambers (Becton-Dickinson Labware, Bedford, MA) with minor modifications to the manufacturer's protocol. Cells were plated at $1.5X10^4$ cells per 24-well culture insert (approximately 70% confluence) for MDA-MB-435 cells, or $2.5X10^4$ cells per well for MCF-7 cells, and allowed to migrate through the Matrigel-coated membrane for 72 hours. Conditioned media from cultured primary human breast fibroblasts was used as a chemoattractant in the migration assay. Cells that had not migrated at the end of the experiment were completely removed from the plating side of the membrane by rubbing with a cotton-tipped applicator. Cells that had migrated to the other side of the membrane were stained with crystal violet reagent and air-dried on the membrane. Membranes were removed and mounted on glass slides using histological mounting media for quantitation of cellular invasion. The number of cells that had migrated was counted under a light microscope using a manual cell counter. Cells that had been induced to overexpress Matriptase by treatment with doxycycline were not found to have altered invasive properties when compared with cells that had not been treated with the drug.

Nude mouse studies:

In order to evaluate the effect of Matriptase overexpression on the growth and invasive properties of MCF-7 cells grown as xenografts in nude mice, we conducted a series of nude mouse experiments. As described in the previous annual report, we had significant difficulty getting the cells to grow well in nude mice, however, we were ultimately able to get several experiments to work.

Animals were handled in accordance with the Georgetown University Animal Care and Use Committee (GUACUC) guides for animal care. MCF-7 cells were cultured to approximately 70% cell density, and removed from tissue culture plastic by gentle scraping using sterile cell scrapers. Scraped cells were pelleted by centrifugation at 500g, and re-suspended in sterile culture media. Viable cells were counted using a hemocytometer, and the concentration of cells adjusted to $5X10^7$ cells per mL. Cell viability was verified by trypan blue exclusion. Using a 25-gauge needle and syringe, $5X10^6$ MCF-7 cells (0.1mL) were implanted by subcutaneous injection into the area of the mammary fat pad between nipples 3 and 4 on each side of NCr nu^-/nu^- (athymic) nude mice. Mice also received a 72 mg/60-day release estrogen pellet (Innovative Research of America, Inc., Sarasota, FL) in a sub-cutaneous, supra-scapular space at the time of tumor inoculation, since MCF-7 cells require estrogen supplementation for their growth in nude mice. Tumor volume was determined by measuring the length, width, and height of tumors and calculating their product. Mice were sacrificed as a cohort at 78 days after tumor inoculation, except for some mice that were sacrificed earlier, due to reproductive system infections. The lungs, liver, kidneys, and brain were removed from mice at the time of sacrifice, and treated with X-gal substrate for 1 hour at room temperature as previously described (Kurebayashi, et al. 1993). Afterwards, organs were fixed by immersion in cold 4% formaldehyde in PBS for 6 hours, and washed several times in cold PBS. Organs were paraffin-embedded, and tissue sections were cut to 5um thickness, stained by H&E, and subsequently examined for metastatic disease.

Tumor dimensions were measured every 7-12 days, and tumor volume calculated by multiplying height by width by length. Mean tumor volume was plotted versus time after tumor inoculation for each cohort.



A descriptive analysis was performed by calculating the mean tumor volumes and their standard errors for each group at each time point. To examine the differences in average tumor growth between the two groups, linear mixed models were fit with correlation to account for repeated measurements from the same mouse. Variation was reduced by using log transformation of the tumor volumes as the response variable, and the explanatory variables include the diet group, measurement time, and their interaction. In the mixed model, the repeated statement was used to model the covariance structure within experimental units (mice). All data analyses were performed in SAS 8.0 (SAS Institute, Inc., Cary, NC).

Examination of tumor growth rate did not show any significant difference ($p=0.39$) between mice fed a standard diet or a doxycycline-containing diet for the induction of matriptase (Figure 3.16 and Table 3.1). Primary tumors grew as stroma-encapsulated masses in both cohorts of mice (data not shown). Therefore the induction of Matriptase expression within primary tumors did not appear to increase the local invasiveness of primary tumors. Removed organs, including lungs, liver, kidneys, and brain, were examined for metastatic disease, determined by the presence or absence of blue cells in H&E stained tissue sections. None were found in any organ for either cohort of mice (data not shown). Together, these results indicate that matriptase induction did not increase the growth of tumors, the local invasion of tumors, nor the metastatic spread of tumor cells.

Comparisons were made between tumor volumes for each group at each time point. Overall, the mean tumor volumes increase with time, and reach the largest volume at day 78 for each of the two groups. The mean tumor volume for the without DOX (Matriptase off) group is always larger than that for the Dox (Matriptase on) group at each time point. From the mixed model, the p value of the test for difference by diet group is 0.39. Hence, there is no statistically significant difference in tumor growth between the two diet groups. Note the large standard errors at each time point.

	Without DOX (Matriptase off)		With DOX (Matriptase on)	
Time after tumor inoculation (days)	# of mice remaining	Tumor volume (mm^3)	# of mice remaining	Tumor volume (mm^3)
7	10	44.86 (2.72*)	10	39.57 (1.72)
17	10	50.38 (3.76)	10	44.43 (3.16)
24	10	63.33 (5.6)	10	63.18 (2.86)
36	10	136.14 (21.43)	10	108.01 (12.75)
44	10	182.73 (33.15)	10	123.64 (23.06)
50	9	188.89 (37.76)	10	108.92 (18.07)

60	9	220.39 (43.62)	10	149.79 (30.25)
70	8	226.01 (48.79)	8	169.77 (38.74)
78	5	259.53 (56.41)	8	237.89 (68.10)

We found the difficulties that we were having getting our cells to grow in nude mice quite troubling. On testing the tumorigenicity of the parental line used to generate our transfectants, we discovered that they did not grow well in nude mice. We were worried that this attenuated behavior might have impacted the results obtained in our studies of the in vitro properties of the cells, rendering them un reliable. We therefore decided to use another MCF-7 line, that had already been transfected with a tet-off transactivation construct, and which we had shown were much more tumorigenic. These cells were transfected with the Matriptase construct and clones in which the protease was highly regulated were isolated. We assayed these clones for the majority of the same parameters used to characterize the initial lines, and found that again, Matriptase overexpression had no effect on the invasiveness, motility, morphology, growth rate or ability to grow in semi-solid media or to degrade matrices. The cells were also used in nude mouse experiments, and Matriptase expression had no effect on the growth of the tumors.

Conclusion:

Thus the goals of the first aim of the proposal have been achieved and we have established that increased Matriptase expression does not appear to impact the malignant behavior of these breast cancer cells. From this several conclusions are possible. The first is that Matriptase expression is irrelevant to the processes he have evaluated and so it is not surprising that altering its expression should have no effect on them. The second is that MCF-7 cells already express more than enough Matriptase to maximally mediate the role it has in these processes and so, again, increasing expression would not be expected to have any impact. The third is that MCF-7 cells make sufficient HAI-1, the endogenous inhibitor of Matriptase, to completely inhibit the relatively modestly increased amount of Matriptase that these cells generate. Thus again, the expected result would be no effect on the behavior of the cells. The data generated in this part of the study cannot determine which of these possibilities is correct. Further studies are ongoing to test which is the more likely scenario and the second task of this project will generate data that will allow us to evaluate the third possibility.

Progress on Task 2

In the previous report we described our progress to ward the goals of Task 2 – to use Ribozyme technology to reduce the level of HAI-1 expression in MCF-7 cells thereby shifting the Matriptase / HAI-1 balance in favor of Matriptase. With the assistance of our collaborator Dr Anton Wellstein, we generated several series of Ribozymes against different portions of the Matriptase gene and tested these in a variety of systems. The problem that we have consistently encountered is developing assay systems to evaluate

the function of the Ribozymes. Simple transient transfection studies did not convince us that the molecules were operating appropriately and so we went on to generate stable expression constructs and used these to stably transfect MCF-7 cells with several different constructs. Again, we were never able to convince ourselves that the constructs were working. Arguing that there might be selection pressure against clones expressing a functional Ribozyme, we went on to generate tetracycline inducible constructs and made cell lines using these. Again, results were equivocal. Since no – regulation system is completely leak-proof, as we had demonstrated in task 1, we decided to go back to evaluation using transient transfection studies this time using a bi-cistronic expression system, with an integral fluorescence tag, so that successfully transfected cells could be identified. Once again, results were variable and not very positive. Having generated an additional series of Ribozymes, we tested them with similar results. We gave up on Ribozymes and generated traditional antisense constructs. Again, none of these produced particularly convincing results.

From all of this, it should be clear that our progress toward the goal of completing task 2 has been minimal – though not through lack of trying. For this reason we requested a no-cost extension of the project to provide an additional year in which to complete the work. We conclude that a part of the difficulty we have been experiencing is due to the cells resistance to expressing Matriptase in the absence of HAI-1. We have tested hundreds of cell lines for the expression of Matriptase and HAI-1 and in the 60 or so lines that we have found that express Matriptase, all without exception express HAI-1 as well. There are, in contrast lines which express HAI-1 in the absence of Matriptase. This is in contrast to our immunohistochemical studies of human tumors in which we have found many tumors that have lost expression of HAI-1 and yet continue to express Matriptase. Thus, we believe that this is an important area that we should continue to try and study, because we believe that the difficulties we have experienced are telling us something important. We believe that there is something in the tissue culture environment that renders unopposed Matriptase toxic to the cells, and so we are exploring models in which we can grow the cells in a more relevant, three-dimensional environment to complete the work. We are in the process of rewriting the statement of work, to cover these studies and will submit a request to allow us to alter the thrust of the second task of the project to allow us to capitalize on this work.

Key Research Accomplishments:

1) We have used an inducible overexpression system to evaluate the effects of Matriptase on a variety of cellular parameters
2) We have demonstrated that Matriptase overexpression has no effect on the ability of cells to grow in soft agar and that matrigel outgrowth morphology is not altered.
3) We have show that the invasive properties of MCF-7 cells are not altered by increased Matriptase expression

4) We have shown that Matriptase overexpression does not impact the ability of MCF-7 cells to form tumors and to proliferate when grown as xenografts in the nude mouse.

5) We have demonstrated that it is very difficult to stably alter the balance of Matriptase to its inhibitor HAI-1

Reportable Outcomes:

Oberst M.D., Johnson M.D., Dickson R.B., Lin C.Y., Singh B., Stewart M., Williams A., al-Nafussi A., Smyth J.F., Gabra H. and Sellar G.C. (2002) Expression of the serine protease Matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. Clin Cancer Res 8:1101-7

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Lin, C.Y., Wang, J.K., Torri, J., Dou, L., Sang, Q.X.A., Dickson, R.B. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. J Biol Chem 272:9147-9152, 1997.

Abbreviations:

βgal	Betagalactosidase
HAI-1	Hepatocyte Activator Inhibitor-1
FITC	Fluorescine Isothiocyanate
MMP	Matrix Metallo Protease